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Protective effect of heat shock proteins: potential for gene therapy

Review Article

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Abbreviations: heat shock proteins, (hsps); herpes simplex virus, (HSV); heat shock transcription factor, (HSF-1); cytokine cardiotrophin-1, (CT-1)

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Summary

The heat shock proteins (hsps) are expressed in normal cells but their expression is enhanced by a number of different stresses including heat and ischaemia. They play important roles in chaperoning the folding of other proteins and in protein degradation. In the heart and the brain, a number of studies have shown that prior induction of the hsps by a mild stress has a protective effect against a more severe stress. Moreover, over-expression of an individual hsp in cardiac or neuronal cells in culture and in the intact heart or brain of either transgenic animals or using virus vectors, also produces a protective effect, directly demonstrating the ability of the hsps to produce protection. These findings indicate the potential importance of developing procedures for elevating hsp expression in a safe and efficient manner in human individuals using either pharmacological or gene therapy procedures.

I. Introduction

Heat shock proteins

It is now over forty years since Ritossa observed that exposure of the larval salary gland of *Drosophila* to elevated temperature resulted in the appearance of new puffs in the giant chromosomes of these cells (Ritossa, 1962). It is now clear that these puffs represent the transcriptional induction of specific genes which encode a group of proteins known as the heat shock proteins (for review see Lindquist and Craig, 1988; Parsell and Lindquist, 1993).

Although originally demonstrated in *Drosophila*, the induction of a small number of heat shock proteins by elevated temperature, is observed in all organisms studied ranging from prokaryotic bacteria to mammals including man. Moreover, this evolutionary conservation extends not only to the existence of the heat shock response in different organisms but also to the induced proteins themselves which are very similar to one another in very different organisms. Thus, the best characterised hsps, hsp90, hsp70, hsp65 and hsp27 (each hsp is named according to its mass in kilodaltons) are induced in response to heat in all organisms studied from bacteria to man and are highly conserved between different species, for example, the hsp90 protein from mammals shows 60% amino acid identity with the corresponding yeast protein and 78% with the *Drosophila* protein (Rebbe et al, 1987). The various hsps and their characteristics are listed in **Table 1**.

Although originally identified on the basis of their induction by elevated temperature and therefore named the heat shock proteins, these proteins are in fact induced by a wide range of stimuli which are potentially damaging to the cell. Such inducers include infections with a wide variety of different viruses (Collins and Hightower, 1982; Khandijan and Turler, 1983; La Thangue and Latchman, 1988), treatment with ethanol (Plesset et al, 1982), steroid hormones (Norton and Latchman, 1989), and amino acid analogues (Li and Laszlo, 1985). Interestingly, hsps are also induced by processes which may occur during human disease, notably exposure of specific cells such as cardiac or neuronal cells to ischaemia or elevated levels of free radicals (Nowak, 1985; Polla, 1988).

The strong evolutionary conservation of the hsps or stress proteins which was discussed above and their induction by a variety of stressful stimuli, indicates that they are likely to have some critical function in the cellular

response to stress. Interestingly, however, many of these proteins are also synthesised by normal unstressed cells with their synthesis being further enhanced upon exposure to stress. For example, hsp90 is one of the most abundant proteins in unstressed cells, constituting approximately 1% of the total protein in mammalian cells even prior to exposure to stress. This has led to the idea that the function of the hsps is one which is required in normal cells but is needed to an even greater extent in stressed cells. This idea is in accordance with the detailed functional studies of individual hsps which, as shown in **Table 1**, have indicated that a number of them have a role in ensuring the correct protein folding of other proteins within the cells, acting as so-called "molecular chaperones" (for review see Ellis, 1990). Thus, for example, hsp90 associates with the steroid receptors, such as the glucocorticoid receptor and keeps them in an inactive form located in the cytoplasm prior to exposure to steroid. Upon steroid treatment, hsp90 dissociates from the receptor which then can move to the nucleus and activate steroid responsive genes.

Clearly, correct protein folding is of importance in normal cells but factors which aid this process will be required at higher level in stressed cells, when for example, stimuli such as elevated temperature result in an increased level of denatured or partially denatured proteins. This idea is also in agreement with findings which indicate that hsps can be induced by treatment of cells with amino acid analogues, which again would induce the formation of abnormally folded proteins (Li and Laszlo, 1985).

Both in normal cells and in stressed cells there will also be a need to degrade proteins which have become abnormally folded and cannot be rescued by the action of chaperone proteins. It is therefore of interest that ubiquitin which plays a critical role in protein turnover by being linked to proteins marked for degradation, is also induced by elevated temperature and is therefore a heat shock protein (see **Table 1**). A further link between the hsps and protein degradation is provided by the observation that inhibition of hsp70 synthesis enhances the cell death which is induced by inhibiting the proteosome which mediates the degradation of ubiquitinated proteins (Robertson et al, 1999).

The idea of the hsps as proteins which are of importance in normal cells but which assume a greater significance in stressed cells, leads logically to the idea that the induction of these proteins by a stressful stimulus is of itself important in assisting the cell to protect itself from stress. In turn, this leads to the idea that the prior induction of the hsps by a mild stress or by some other non-stressful procedure, would be protective against subsequent more severe stress. This idea obviously has considerable medical importance and has therefore been intensively investigated.

Over the years, this work has effectively proceeded in three stages. Firstly, the demonstration that exposure to mildly stressful stimuli which can induce hsp expression, can in turn protect cells against exposure to a more severe stress. Clearly, such findings implicate the hsps as being protective but do not prove this, since the protective effect could be due to some other action of the mildly stressful treatment, other than its ability to induce the hsps. This idea leads directly to the second stage of these investigations, namely, the use of gene constructs to over-express the hsps in cultured cells and then demonstrate a protective effect against subsequent exposure to stress. Finally, more recently, these experiments in cultured cells have been complemented by experiments over-expressing the hsps in an intact animal and again demonstrating a protective effect. In subsequent sections of this review, I will discuss these three stages of work on the protective effect of heat shock proteins, focusing on studies involving neuronal or cardiac cells in culture or in the intact brain and heart, because of the key medical importance of these organs.

Table 1. Major eukaryotic hsps

Family	**Members**	**Prokaryotic Homologue**	**Functional Role**	**Comments**
Hsp90	Hsp100, Hsp90 Grp94	C62.5 (*E. coli*)	Maintenance of proteins such as steroid receptor. Src. in an inactive form until appropriate	*Drosophila* and yeast homologues of hsp90 are known as hsp83
Hsp70	Grp78 (= Bip) Hsp72, Hsp73 Hsx70	dna K (*E. coli*)	Protein folding and unfolding: assembly of multimeric complexes	Hsx-70 only in primates
Hsp65	Hsp65	gro EL (*E. coli*) Mycobacterial 65 kd antigen	Protein folding and unfolding: organelle translocation	Major antigen of many bacteria and parasites which infect man

Hsp56	Hsp56	-	Protein folding, component of steroid receptor complex	Binds FK506 (tarcolimus) and is also known as FKBP56
Hsp32	Hsp32	-	Cleaves heme to yield carbon monoxide and the protective anti-oxidant molecule, biliverdin	Also know as heme oxygenase-1
Hsp27	Hsp27, Hsp26, etc.	Mycobacterial 18 kd antigen	Unclear	Very variable in size and number in different organisms
Ubiquitin	Ubiquitin	None	Protein degradation	Also conjugated to histone H2A in the nucleus leading to potential role in gene regulation

II. Protective effect of stimuli which induce hsp synthesis

During the 1980s, a very large number of studies demonstrated that, in cells in culture, stimuli which induced hsp synthesis such as a mild stress resulted in protection against subsequent exposure to a more severe stress. Moreover, it was also demonstrated that the levels of the hsps induced by such mildly stressful procedures, generally correlated with the level of protection which was observed against the subsequent more severe stress (for review see Lindquist and Craig, 1988; Parsell and Lindquist, 1993).

Following such early studies primarily carried out in cell lines of fibroblast origin, this work was extended also by carrying out similar studies both in cell lines of neuronal origin and in primary neuronal cells. Thus, for example, primary neuronal cells cultured *in vitro* are protected by exposure to mild heat or ischaemic stress from a subsequent more severe heat or ischaemic stress or exposure to the excitotoxin glutamate (Lowenstein et al, 1991; Rordorf et al, 1991; Amin et al, 1995). Indeed, in our own studies, the degree of protection afforded by an initial mild stress correlated with the amount of hsp induced, rather than the nature of the subsequent stress. Thus, a mild heat stress produced a better protective effect against subsequent severe heat stress or severe ischaemia, than was observed for a mild ischaemic stress correlating with the greater degree of hsp induction produced by the mild heat stress (Amin et al, 1995).

These *in vitro* studies examining the protective effect of a mild stress against a more severe stress, have also been supplemented by examining the protective effect of such mild stresses against exposure to stimuli which induce apoptosis (programmed cell death). Thus, it has been shown that prior mild heat shock can protected the ND7 neuronal cell line against apoptosis induced by serum withdrawal and addition of retinoic acid (Mailhos et al, 1993). Similarly, primary neonatal dorsal root ganglion cells are protected by mild heat shock against subsequent withdrawal of nerve growth factor, which induces apoptosis in these neurones (Mailhos et al, 1994).

These *in vitro* observations were subsequently extended by studies in the intact animal *in vivo*. For example, prior exposure of the animal to a mild heat stress is sufficient to protect retinal neurones against a subsequent *in vivo* exposure to either light damage (Barbe et al, 1988) or ischaemia (Chopp et al, 1989). Similarly, exposure to a mild cerebral ischaemia protects hippocampal neurones against subsequent exposure to a more severe ischaemia (Kitagawa et al, 1990).

Similar studies have also been carried out in the whole heart either perfused *ex vivo* or in the intact animal *in vivo*, demonstrating that stimuli which result in hsp induction can protect the heart against subsequent exposure to a more severe stress. This was first demonstrated by Currie et al, (1988) who exposed rats to elevated temperature and then removed their hearts and exposed them to ischaemia on a Langendorff perfusion apparatus. They demonstrated that the hearts from rats which had been exposed to an elevated temperature showed improved recovery of contractile function following subsequent ischaemia and reperfusion compared to control hearts. Furthermore, the reperfusion damage, as measured by creatine kinase release was significantly reduced in the heat shock hearts. These findings therefore demonstrated for the first time that a stimulus which induced hsp induction in the intact heart was able to

produce a protective effect against subsequent exposure to ischaemia/reperfusion. These results have subsequently been extended both by examining other parameters of heart function and by using other species such as the rabbit (Karmazyn et al, 1990; Yellon et al, 1992) (for review see Yellon and Latchman, 1992).

These studies demonstrating a protective effect in the heart on a Langerdorff perfusion apparatus following prior exposure to elevated temperature *in vivo*, lead naturally to the question of whether a similar protective effect would be observed in hearts exposed to myocardial ischaemia within the intact animal following a prior exposure to heat shock.

Donnelly et al, (1992) demonstrated that this was indeed the case with an effective reduction of infarct size being observed when rat hearts were exposed to 35 minutes of left coronary artery occlusion in the intact animal following exposure to heat shock. Moreover, this protective effect is not confined to the use of heat shock itself to induce the hsps. Thus, Marber et al, (1993) were able to demonstrate that four brief periods (5 minutes each) of cardiac ischaemia were able to induce hsp synthesis and were also able to reduce infarct size when the hearts were subsequently exposed to 30 minutes of ischaemia in the intact animal.

Hence, stimuli which result in hsp induction in the intact heart *in vivo* can produce a protective effect against subsequent exposure of the heart to ischaemia/reperfusion either on a perfusion apparatus or within the intact animal. In addition, a number of studies have demonstrated that the protective effect correlates with the amount of heat shock protein which is induced. Thus, for example, Marber et al, (1994) showed a correlation between the amount of hsp70 produced by heat stress of papillary muscle and the muscle's ability to recover function following a period of hypoxia. Similarly, Hutter et al, (1994) demonstrated a similar correlation between the amount of hsp70 and the ability to limit infarct size following exposure of the heart to ischaemia and subsequent reperfusion.

These studies indicate therefore, that neuronal and cardiac cells can be protected by prior exposure to a mild stress sufficient to induce hsp over-expression. Moreover, the correlation between the amount of hsp induced and the degree of protection observed, suggests that it is the induction of the hsp rather than some other effect of the mild stress, which produces the protective effect against the more severe stress. However, such studies are essentially only correlative and to prove that hsps can have a protective effect, it is necessary to over-express individual hsps in neuronal or cardiac cells. Such studies are discussed in the next section.

III. Protective effect of individual hsps in neuronal and cardiac cells *in vitro*

In a number of cases, it has been possible to show that over-expression of an individual hsp can provide a protective effect against damaging stimuli, in the same manner as a mild hsp-inducing stress. Thus, for example, dorsal root ganglion neurones can be protected against thermal or ischaemic stress by over-expression of either hsp70 or hsp90 (Uney et al, 1993; Amin et al, 1996; Wyatt et al, 1996) and a similar effect of hsp70 and hsp90 has been observed in the ND7 neuronal cell line (Mailhos et al, 1994). Similarly, Fink et al, (1997) were able to protect cultured hippocampal neurones against subsequent heat shock using a herpes simplex virus (HSV)-derived amplicon vector expressing hsp70, indicating that this effect applies to neurones derived from both the central and the peripheral nervous systems.

Similar studies on the protective effect of the hsps in cardiac cells, initially focused on hsp70 and utilised the H9c2 cell line which was derived initially from the rat heart. In 1994, two groups reported the results of experiments in which stable transfection was used to produce clonal cell lines derived from H9c2 which constitutively over-expressed hsp70 (Heads et al, 1994; Mestril et al, 1994). These cells were shown to be protected against subsequent exposure to thermal or ischaemic stress compared to control cells which did not over-express hsp70. These studies were subsequently extended by Cumming et al, (1996b) who demonstrated that similar protective effects against heat stress or simulated ischaemia could be observed when hsp70 was over-expressed by transfection of primary rat cardiac myocyte cultures, demonstrating that this protective effect could be observed both in primary cardiac cells and in cell lines derived from them. A similar protective effect was also observed when hsp70 was over-expressed by transfection in coronary endothelial cells (Suzuki et al, 1998) indicating that hsp70 can protect these cells as well as cardiac myocytes. This is of particular interest since it has been shown that when the heart is exposed to elevated temperature *in vivo*, hsp70 induction occurs primarily in endothelial cells rather than in cardiac myocytes (Amrani et al, 1998; Leger et al, 2000).

To extend these experiments to other hsps, transfection methods were used to over-express hsp90, hsp65, or hsp56 either in the H9c2 cell line (Heads et al, 1995) or in cultured primary cardiac cells (Cumming et al, 1996a,b). In these experiments, hsp90 over-expression was able to protect the cells against subsequent thermal stress but not against subsequent simulated ischaemia whereas hsp65 or hsp56 had no protective effect. Since hsp70 over-expression protected against both thermal or simulated ischaemic stress in these experiments, these studies indicate that different hsps can have different protective effects and need to be tested individually for their protective effect in any specific situation.

This idea is reinforced by findings in neuronal cells where the over-expression of an individual hsp does not always reproduce the protective effect of a mild hsp-inducing stress. Thus, over-expression of hsp70 or hsp90 in ND7 cells (Mailhos et al, 1994) or DRG neurones (Wyatt et al, 1996) does not reproduce the protective effect of mild heat shock against subsequent apoptotic stimuli. Similarly, in the experiments of Fink et al, (1997) over-expression of hsp70 with an hsp vector did not protect hippocampal neurones against glutamate toxicity which may act by inducing apoptosis (Kure et al, 1991), despite the fact that previous studies demonstrated a clear

protective effect of mild heat stress against subsequent exposure to glutamate (Lowenstein et al, 1991; Rordorf et al, 1991).

However, in further experiments we were able to show that the protective effect of a mild heat stress against apoptotic stimuli in neuronal cells could be reproduced by over-expressing the small heat shock protein hsp27. Thus, over-expression of hsp27 using an herpes simplex virus (HSV-)based vector was able to protect both ND7 cells and DRG neurones against apoptosis induced by withdrawal of serum or nerve growth factor, whereas such protection was not observed when hsp70 was over-expressed with a similar vector (Wagstaff et al, 1999) (**Figure 1a**). As expected, over-expression of either hsp27 or hsp70 by this means was able to protect the neuronal cells against subsequent exposure to heat shock or ischaemia, paralleling the results obtained with plasmid constructs for hsp70 and extending this to hsp27 (Wagstaff et al, 1999).

Interestingly, when these experiments with HSV vectors over-expressing individual hsps were used to determine their protective effect in primary cardiac cells (Brar et al, 1999), we confirmed our earlier results that hsp70 over-expression can protect cardiac cells against simulated ischaemia or thermal stress, whereas over-expression of hsp56 has no such protective effect. Moreover, we were able to extend these studies by showing firstly, that hsp70 can protect against the induction of apoptosis (programmed cell death) in cardiac cells by exposure to ceramide, whereas hsp56 has no protective effect and secondly, to demonstrate that over-expression of hsp27 (which we had not previously tested) similarly protects cardiac cells against subsequent exposure to thermal or ischaemic stress or to ceramide (**Figure 1b**). Hence, in cardiac cells both hsp70 and hsp27 can protect against apoptosis whereas in neuronal cells only hsp27 has this protective effect. This reinforces the need to study individual hsps for their protective effect against specific stimuli and in specific cell types.

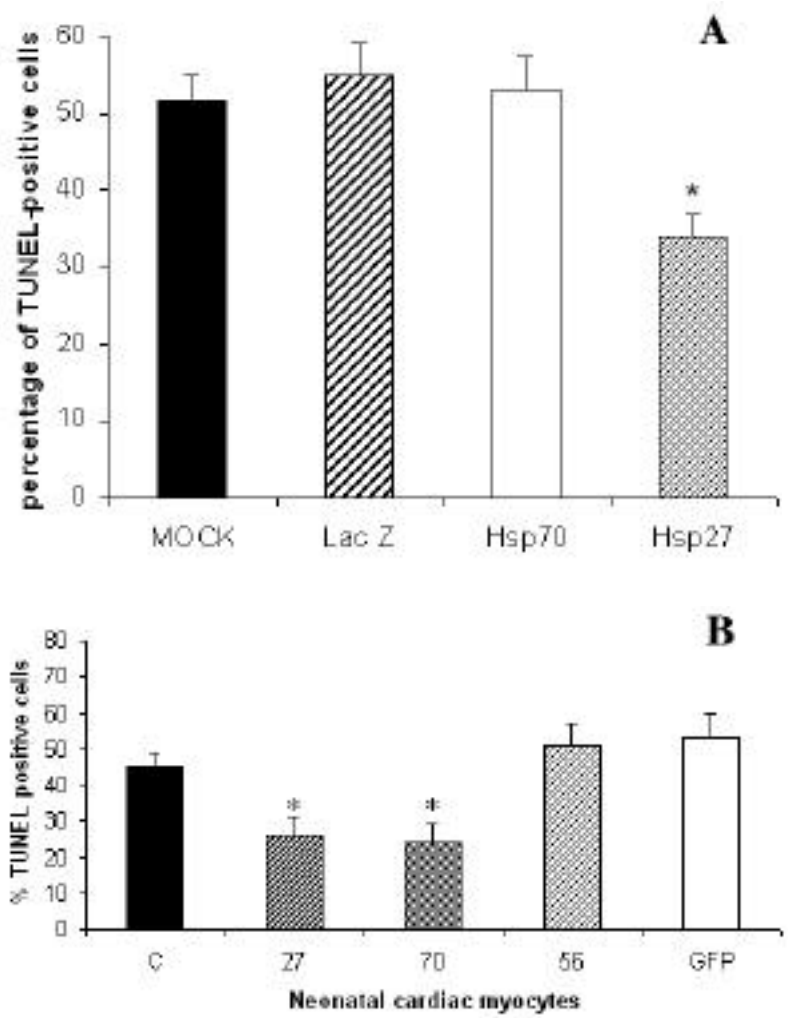

Figure 1. (**A**) Number of DRG neurones undergoing apoptosis (as assayed by TUNEL staining) after NGF withdrawal following prior infection with the indicated virus. Values are the mean of three determinations whose standard error is shown by the bars. Significant enhancement of survival ($p < 0.05$) was observed only with hsp27-expressing virus. (**B**) Percentage of apoptotic cells (as assayed by TUNEL staining) in cardiomyocytes pre-infected with the indicated viruses or left untreated (C) and then 24 hours after infection either left untreated or treated for six hours with 25μM ceramide. The data represent the means of two independent experiments whose standard error is indicated by the bars. Both hsp27 and hsp70-expressing viruses significantly reduced the number of apoptotic cells compared to uninfected cells (C) or cells infected with a control virus expressing green fluorescent protein (GFP) ($p < 0.05$).

These findings also suggest that hsp27 may be as protective as hsp70 in cardiac cells whilst potentially being more protective in neuronal cells. Similar results were also obtained by Martin et al, (1997; 1999) who used an adenovirus vector to over-express hsp27 or the related protein αB-crystallin in cardiac cells. They were able to demonstrate that both these proteins were able to protect cardiac myocytes from the effect of simulated ischaemia and that decreasing the level of endogenous hsp27 using an antisense approach enhanced the damaging effects of a subsequent ischaemic stimulus.

Taken together therefore, these results demonstrate that several hsps can play an important role in protecting cells in culture from the effects of damaging stimuli. They therefore raise the possibility that the ultimate use of hsps in therapeutic procedures (see below) may be optimised by stimulating the over-expression of more than one hsp to produce an optimal protective effect. This is reinforced by the findings of Lau et al (1997) who were unable to demonstrate any protective effect of over-expressing hsp60 or hsp10 individually in cultured cardiac cells but did observe a protective effect when both proteins were over-expressed together.

Hence, these studies do clearly demonstrate that over-expression of individual hsps can protect cultured neuronal or cardiac cells against different death-inducing stimuli, extending the results with prior mild hsp-inducing stresses. Moreover, they demonstrate that the protective effect of individual hsps may vary with the cell type being investigated and the nature of the stress being used and also, provide the first suggestion that hsp27 may have a more potent protective effect than hsp70 in the nervous system. These findings reinforce the need to extend these *in vitro* studies on over-expression of the hsps to their over-expression in the intact animal. Such studies are discussed in the next section.

IV. Protective effect of hsps *in vivo*

In keeping with the strong focus in the hsp field on the major inducible hsp, hsp70, transgenic animals over-expressing this hsp were reported by several groups in 1995-96 (Marber et al, 1995; Plumier et al, 1995; Radford et al, 1996). In their initial analysis of these mice, all these groups focused primarily on the potential protective effect of hsp70 in the heart. In all cases, they were able to demonstrate that such over-expression of hsp70 was able to protect the heart against the damaging effects of ischaemia using a variety of assays such as infarct size, creatine kinase release, recovery of high-energy phosphate stores and correction of metabolic acidosis. Moreover, in a subsequent study, it was demonstrated that such a protective effect could also be observed against myocardial dysfunction caused by a brief ischaemia which was insufficient to produce an infarct (Trost et al, 1998).

These studies thus establish for the first time, that the over-expression of a single hsp *in vivo* in the intact animal is sufficient to protect a specific organ, namely the heart, against the damaging effects of a stressful stimulus. In view of the clear evidence demonstrating that there is also a protective effect for the hsps in neuronal cells *in vitro* (see above), it is not surprising that these hsp70 transgenic animals were rapidly used in attempts to demonstrate that over-expression of hsp70 would also protect the brain of the intact animal from specific stresses. In general, however, the results of these studies have been far more equivocal than the corresponding studies in the heart, with protection being observed against some insults but not against others (Plumier et al, 1997; Rajdev et al, 2000; Lee et al, 2001). Thus, for example, Lee et al, (2001) found that one of the hsp70 transgenic mouse strains showed no reduction of infarct size or enhanced survival of neuronal cells following cerebral ischaemia and similar results were also obtained using a different strain by Plumier et al, (1997) in terms of infarct size and striatal neurone survival, although they did observe enhanced survival of hippocampal neurones.

In view of these results and the apparent highly potent protective effect of hsp27 in cultured neuronal cells, we have recently prepared the first transgenic mice over-expressing hsp27 in the brain and other tissues (Akbar et al, 2003). Most interestingly, the hsp27 over-expressing transgenic animals showed very clear protective effects when treated with kainate, which normally induces considerable cell death in the CA3 region of the hippocampus. Thus, whilst control wild type litter-mates showed a 33% cell loss in this region when treated with kainate compared to control untreated animals, no significant cell death was observed in the kainate-treated transgenic animals compared to untreated transgenic animals. Moreover, this effect at the cellular level was accompanied by a significant effect on reducing mortality in the hsp27 over-expressing animals, with on average 17% of hsp27 transgenic mice dying following kainate treatment compared to 38% of wild type mice, similarly treated.

As well as these effects on survival, both at the whole animal and the individual neurone level, the hsp27 mice also showed much milder seizures throughout the four-hour observation period following kainate treatment, compared with the wild type animals (**Figure 2**).

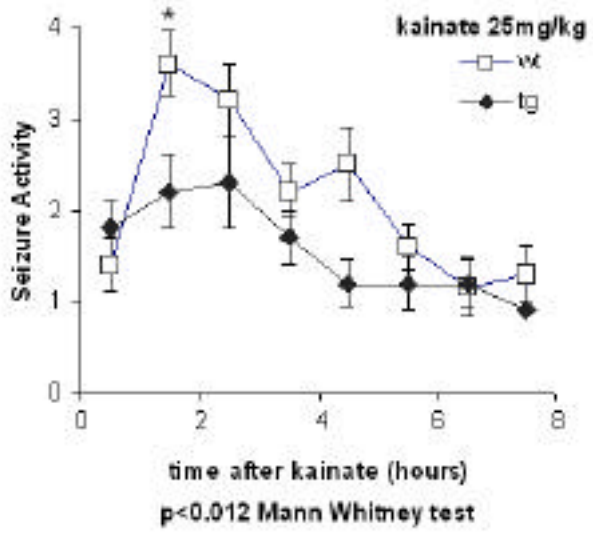

Figure 2. Reduced seizure activity in hsp27 transgenic mice (tg) compared to wild type mice (wt) following kainate treatment.

Hence, hsp27 over-expression has a clear potent protective effect in the nervous system in terms of kainate toxicity, both at the level of whole animal seizure activity and survival and at the level of vulnerable neurones within the hippocampus (Akbar et al, 2003). It will evidently be of considerable interest to extend this study by determining whether hsp27 can have protective effects against other damaging stimuli in the nervous system including, for example, cerebral ischaemia. It will also be of interest to conduct a detailed study of the hsp27 and hsp70 over-expressing transgenic animals, to compare the potency of these different heat shock proteins against various damaging stimuli in both the heart and the brain and to confirm or deny the suggestion that hsp27 may be more potent than hsp70 in protecting the nervous system.

It is already clear however, that hsp27 does have a clear protective effect in the nervous system *in vivo* as well as *in vitro*. This reinforces the need to conduct studies using different individual hsps and investigating their protective effects, rather than simply focusing on the major heat shock protein hsp70.

V. Therapeutic potential of hsps

The experiments described in earlier sections, clearly suggest that procedures which elevate hsp levels in the heart or the brain may be of significant benefit, for example, during reperfusion following a period of ischaemia, or in patients undergoing neuronal cell loss due to neurodegenerative diseases such as Alzheimer's or Parkinson's diseases. Similarly, elevation of hsp levels may be beneficial during cardiac bypass or to preserve donor heart function prior to transplantation. Indeed, in view of the use of cold storage during transportation prior to transplantation, it is of particular interest that reduced as well as elevated temperature induces hsp expression in the heart (Laios et al, 1997). Moreover, a mild heat treatment prior to hypothermic storage has been shown to enhance subsequent recovery of the heart (Gowda et al, 1998a).

Such temperature-based manipulations of the hsps may thus have a role to play in cardiac transplantation procedures. Similarly, it has been shown that a protective effect across the whole heart can be obtained by using a thermal probe to produce local heating of the heart (Gouda et al, 1998b) suggesting that a similar procedure could be used therapeutically.

The induction of the hsps by stressful stimuli such as elevated temperature (for review see Morimoto, 1998) or ischaemia (Nishizawa et al, 1999) is mediated by the heat shock transcription factor (HSF-1). Thus following exposure to elevated temperature, the cytoplasmic HSF-1 monomer, forms a trimer and moves to the nucleus where it binds to its target sites (known as heat shock elements) in the regulatory regions of the hsp genes and, following HSF-1 phosphorylation, it induces hsp gene expression.

Interestingly, the induction of the hsps by stressful stimuli diminishes with age in a variety of tissues including the heart and this has been shown to be due to impaired activation of HSF-1 by stress in the aged heart (Locke and Tanguay, 1996). Moreover, this effect is associated with a reduced protective effect of mild heat shock or ischaemia against a subsequent severe ischaemic stress in aged hearts (Locke and Tanguay, 1996; Fenton et al, 2000). As many of the situations where the protective effect of hsps would be valuable involve elderly individuals, this suggest that other procedures not involving stressful stimuli or HSF-1 may be required for the therapeutic induction of the hsps.

Obviously, such an approach would be dependent on over-expression of the hsps actually having a protective effect in aged cells. To test this, we have recently used our HSV vectors to over-express individual hsps in neurones from aged rats or peripheral blood lymphocytes from aged humans. These experiments (Alsbury et al, submitted) clearly showed that hsps do have a protective effect in cells from aged humans or animals, if they are successfully over-expressed. Hence, it is indeed appropriate to try and identify procedures which result in over-expression of the hsps in a non-stressful manner. Such procedures can be divided into pharmacological and gene therapy procedures.

A. Pharmacological methods

Although the hsps were identified on the basis of their induction by stressful procedures, they are also induced naturally by specific non-stressful procedures (for review see Latchman, 1998). For example, the cytokines interleukin-6 (Stephanou et al, 1997) and interleukin-10 (Ripley et al, 1999) can induce hsp gene expression in a non-stressful manner. It has been shown that these inducers do not act via HSF-1 but activate hsp expression via other transcription factors such as NF-IL6 and STAT3 (for review see Stephanou and Latchman, 1999). Based on the use of these inducers in non-cardiac cells, we demonstrated that the interleukin-6-like cytokine cardiotrophin-1 (CT-1) was able to induce hsp synthesis in cultured cardiac cells and that such treatment protects them against subsequent exposure to severe thermal or ischaemic stress (Stephanou et al, 1998). Similar induction of the hsps in cultured cardiac cells and protection against subsequent severe stress is also observed with the tyrosine kinase inhibitor herbimycin A (Morris et al, 1996; Conde et al, 1997).

These protective effects in cardiac cells in culture have also been extended to the intact heart. For example, Vigh et al, (1997) showed that bimoclomol, a novel hydroxylamine derivative was able to induce hsp synthesis in the intact perfused heart *ex vivo* and to produce a protective effect against a subsequent ischaemia. Similarly, Meng et al, (1996) demonstrated that norepinephrine treatment of an intact rat resulted in hsp induction in the heart and protection against ischaemia when the heart was subsequently perfused *ex vivo*.

Several compounds thus exist which can induce hsps and produce a protective effect, although it should be note that in no case has this protective effect been directly shown to be due to the ability to induce hsps. Before the protective effect of any of these compounds could be exploited clinically, it is evidently necessary to investigate whether their protective effect in the heart can be achieved without any significant side-effects. For example, CT-1 was originally identified on the basis of its ability to induce cardiac hypertrophy (Pennica et al, 1995) whilst

herbimycin as a tyrosine kinase inhibitor is likely to have significant effects on cellular growth and division.

Similarly, none of these compounds has as yet been tested to see whether, unlike stressful procedures, they can induce hsps in cells from aged animals or humans. This is of particular importance in the case of bimoclomol, which appears to be non-toxic but which has recently been shown to act by targeting HSF-1 (Hargitai et al, 2003). Nonetheless, the identification of compounds able to induce the hsps without inducing a full stress response is highly promising and suggests that pharmacological induction of hsp synthesis may be a viable therapy in the not too distant future.

B. Gene therapy procedures

Clearly any potential side-effects of compounds which can induce hsps could be avoided if hsp genes could be efficiently delivered to the heart or the brain *in vivo*. Since transgenic procedures are evidently not applicable in humans, this will require the development of procedures able to safely and efficiently deliver hsp genes to the heart of individual patients.

Encouragingly, it has been shown that the hsp70 gene within a plasmid vector can be delivered to the heart via intra-coronary infusion of liposome particles containing it. The elevated expression of hsp70 produced by this means confers effective protection against subsequent ischaemia-reperfusion (Suzuki et al, 1997) or endotoxin-induced cardiac damage (Meldrum et al, 1999). These experiments are of considerable importance since they demonstrate that hsp70 over-expression can have a protective effect not only in a transgenic animal but also in a situation directly relevant to the human case where hsp over-expression is produced in the adult heart by introduction of the gene construct.

As well as these experiments in the heart, similar successful delivery of hsps to the intact nervous system has been reported. Indeed, Benn et al, (2002) have used an adenovirus vector over-expressing hsp27 to rescue sciatic motor neurones in the intact animal from the cell death which normally follows nerve transection. Similarly, using our HSV vector over-expressing hsp27, we have demonstrated a protective effect of inoculation of the brain with this virus against kainate-induced cell death (Kalwy et al, 2003), reproducing the protective effect of hsp27 in transgenic animals.

If these reports of the successful *in vivo* delivery and protective effect of hsps can be followed up using viral or non-viral vectors which combine sufficient efficiency with the safety required for human use, then it may be possible to contemplate gene therapy-type procedures with hsps.

The results presented in this review however, indicate that it is necessary to develop such procedures always bearing in mind the fact that the protective effect of hsps can differ in different cell types and in different situations. Any attempt to use the hsps therapeutically therefore, has to be preceded by careful studies identifying the optimal hsp or combination of hsps to produce a protective effect in a particular situation. This is illustrated, for example, by our experiments using a constitutively active form of the heat shock transcription factor, HSF1, which should induce a range of hsps. When an HSV vector expressing this HSF1 mutant was used to infect neuronal cells, it induced accumulation of hsp70 but not of hsp27 and therefore, did not result in protection against apoptosis, although it was able to produce protection against heat shock and ischaemia (Wagstaff et al, 1998).

Nonetheless, the clear protective effect of the hsps in the cardiac and nervous systems offers hope for therapeutic procedures which enhance endogenous or exogenous hsp expression.

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